IN VITRO, In Press

Revised Manuscript #861210

MAINTENANCE OF HIGHLY CONTRACTILE TISSUE-CULTURED AVIAN SKELETAL MYOTUBES IN COLLAGEN GEL

Herman H. Vandenburgh[1,2]

Patricia Karlisch[2]

and Lynne Farr[2]

[1]Department of Pathology

Brown University

and

[2]Department of Laboratory Medicine

The Miriam Hospital

Providence, RI 02906

Running Head Title - Myotube Growth in Collagen Gel

Proof and Reprints to:

Dr. H.H. Vandenburgh

Department of Laboratory Medicine

The Miriam Hospital

164 Summit Avenue

Providence, RI 02906

Summary:

Highly contractile skeletal myotubes differentiated in tissue culture are normally difficult to maintain on collagen-coated tissue culture dishes for extended periods of time because of their propensity to detach as a sheet of cells from their substratum. This detachment results in the release of mechanical tension in the growing cell "sheet" and consequently, loss of cellular protein. We have developed a simple method of culturing high density contractile primary avian myotubes embedded in a collagen gel matrix (collagel) attached to either a stainless steel mesh or nylon support structure. With this system the cells can be maintained in a highly contractile state for extended periods of time in vitro under tension. Structural integrity of the myotubes can be maintained for up to 10 days in basal medium without serum or embryo extract if the cells are embedded in collagel. Total cellular protein and myosin heavy chain accumulation in the cells can be maintained for weeks at levels which are 2-3 times those found in time-matched controls which are under little tension. Morphologically, the myotubes are well differentiated with the structural characteristics of neonatal myofibers. This new collagel culture system should prove useful in the analysis of in vitro gene expression during myotube to myofiber differentiation and its regulation by various environmental factors such as medium growth factors, innervation, and mechanical activity.

Introduction:

Differentiation and development of skeletal muscle *in vitro* have been extensively studied and seem to closely follow the *in vivo* situation in many respects, including the fusion of the myogenic cells into multinucleated myotubes (19,27), and the progressive increase in the synthesis (3,7) and organization (11) of muscle-specific proteins. However, removing these cells from their *in vivo* environment and placing them under the artificial conditions associated with tissue culturing isolates them from many important influences needed for complete differentiation into the adult tissue. For example, innervation and increasing mechanical tension during development are several important factors missing from the normal tissue culture situation. These missing components appear to be required for the subsequent complex developmental events resulting in the transition of fetal skeletal muscle into adult muscle fibers (5). Thus, with few exceptions (9), myotubes differentiated in tissue culture without these environmental regulatory factors do not express many of the adult genes, including those for the myofibrillar proteins (4).

High density cultures of highly contractile skeletal myotubes which could be maintained for extended periods of time would assist in the analysis of the regulatory factors involved in the fetal to adult transition in gene expression. Unfortunately, under normal culture conditions, the spontaneously contractile myotubes are difficult to maintain because of their propensity to detach from the substratum. The cultures can be maintained for long periods by culturing at low density or in the presence of drugs which inhibit myotube contraction such as ouabain or tetrodotoxin; but these conditions have a marked effect on the growth of the cells (31) and on their ability to accum-

ulate myofibrillar protein (32). A simplified culture system for maintaining high density, highly contractile myotubes for extended periods of time is described in this paper. A collagen substratum is normally used in skeletal muscle primary cultures to assist in the formation of multinucleated myotubes from precursor myogenic cells (8,15). We have found that by embedding the formed myofibers in a collagen gel matrix which is attached to a stainless steel mesh or nylon support ring assists in the long-term maintenance of the contractile cells and in their more complete structural development. Similar beneficial effects of embedding other cell types in collagen gels have been reported (2,6,13,31). This modified culture system should assist in the analysis of the regulatory factors involved in skeletal muscle terminal differentiation. Parts of this work have appeared in abstract form.[1]

[1] In Vitro Cellular and Developmental Biology 23:24a, 1987.

Methods:

Cell Culture -

Embryonic avian skeletal muscle cells are isolated from 11-12 day *in ovo* pectoralis muscle by standard dissection techniques (28). Fertilized eggs were obtained from Beaver River Farm, W. Kingstown, RI. Briefly, after teasing the muscle tissue away from the skin and underlying bone in Earle's Balance Salt Solution (EBSS) under sterile conditions, the muscle is minced into pieces of approximately 1 mm^2. After accumulating tissue from 3-6 embryos, it is centrifuged at 1,600 xg for 1 min, transferred to 10 ml 0.25% trypsin in Ca^{++} Mg^{++} free EBSS, and incubated for 15 min at 37°C. The cells are again pelleted by centrifugation at 1,500 xg for 5-6 min and transferred to 10 ml of growth medium. Following a 45 sec vortexing at low speed, the dispersed cells are passed through a 20-30 μm Nitex filter. The cell density of the resulting cell preparation is determined with a Levy Chamber and approximately 5,700 cells/mm^2 are plated in 1 ml of growth medium in each well of 4-well tissue culture plates (Nunc Corp., Roskilde, Denmark). The wells are previously collagen-coated by salt-precipitation (20). The cultures are maintained in a humidified 5.0% CO_2 incubator at 37.2°C. Growth medium is Eagle's Basal Medium, 10% pre-tested horse serum (Hyclone Laboratories, Logan, Utah), 5% chicken embryo extract (85/10/5 medium), and 50 units/ml penicillin. All other tissue culture reagents were obtained from GIBCO. Streptomycin is not used because of its deleterious effect on phenotypic maturation of chick myogenic cells (23). All cultures are fed fresh media (500 μl) at 24, 48, and 72 hrs, and then subsequently, as outlined in the Results. Under these growth conditions, the myogenic cells proliferate and fuse during the first 48 hrs *in vitro* and are contractile by 96-120 hrs.

Collagen Gels -

The 15 mm diameter wells in which the myotubes are to be embedded in collagen are prepared prior to cell plating by attaching either a flat nylon washer (13 mm outside diameter, 2 mm wide, purchased from a local hardware store) to the bottom of the collagen-coated wells using RTV Silicone Rubber Adhesive (General Electric, Silicone Products Dept., New York) or by placing a stainless steel mesh (8 mm x 50 mm, no. 100, Newark Wire Cloth Co., Newark, N.J.) around the outside wall of the wells. The mesh and washers were soaked in an Alconox wash solution, rinsed well with warm tap water, and distilled/deionized water before use. Each 4-well tissue culture dish contains 2 wells with mesh or rings (to be collagen-embedded) and 2 normal wells to be used as time-matched controls. All dishes are gas sterilized before use.

Seventy-two hours after plating the cells, the growth medium is removed from the fused myotube cultures, and 500 μl of a collagen gel solution is rapidly added to the wells. The collagen gel solution is prepared immediately before use by adding 0.1 N NaOH to 85/10/5 (250 μl to 9.0 ml) and then 4.5 ml of a collagen solution (250 μg/ml) in acetic acid, prepared according to the method of Bell et al. (2). The solution is rapidly mixed by inversion and pipetted into each well containing a nylon ring or wire mesh. Control cultures receive 500 μl of a similar solution made up with an acetic acid solution not containing collagen. In order for the collagen solution to gel properly, the pH of the final mix is critical. The phenol red indicator in the solution should be slightly yellowish (pH 6.9-7.0) prior to its addition to the cells. Since the particular batch of 85/10/5 being used can affect the mixture's pH, we always premix the collagen gel solution in a test tube and incubate it for 1 hr at 37°C to see if it gels properly. Small changes in the amount of the

sodium hydroxide solution can be made to obtain the proper pH for getting the final solution to gel properly. In more recent experiments, commercially available collagen (Type I Rat Tail collagen, Collaborative Research) has been successfully used. At dilute collagen levels (50-200 μg/ml,) use of NaOH is not required when using the Collaborative Research product.

Following addition of the collagen gel solution to the cells, the dishes are carefully returned to the incubator and left undisturbed for 18-36 hrs. Proper hardening of the gel can be determined by gently tipping the culture dishes and watching to see if the gel has solidified. Once hardened, 250 μl of fresh 85/10/5 is carefully added to the top of the collagen gel. A similar amount of the medium is added to the controls. Subsequent feeding schedules for the cultures are described in the Results. When the cultures are to be fed, the old medium is carefully removed by hand aspiration before the addition of fresh medium. It is critical that the collagen gel is not disturbed unnecessarily during the first 4-7 days prior to its dehydration and collapse onto the cells (see Results).

Chemical Determinations

At varying times, the cultures are removed from the incubator and rinsed three times with phenol-red free EBSS for 2-3 min with swirling at room temperature. Following aspiration of the final rinse solution, the cells are frozen at -20°C until termination of the experiment. At that time, the cells are homogenized by sonication in 500 μl sucrose buffer (0.25 M sucrose, 0.02 M KCl, pH 6.8) on ice and aliquots taken for the determination of total protein (22), total DNA (17), and myosin heavy chain accumulation (29). The fluorometric DNA assay procedure is slightly modified from the procedure of Lubarca and Paigen (21) by eliminating EDTA from the homogenization buffer but includ-

ing it in the phosphate buffer used in the second step of the assay. This is necessary so that an aliquot from the cell homogenate can be used for myosin heavy chain quantitation. Quantitation of myosin heavy chain is accomplished by making an aliquot from this homogenate 2% in SDS, 5% in 2-mercaptoethanol, 20% in glycerol, and 0.0625 M in TRIS-HCl, pH 6.8 and boiling the sample for 2-4 min. Electrophoretic separation of the myosin heavy chains is performed on 5% polyacrylamide gels (1.5 mm thick slabs) by the method of Patterson and Strohman (25). Quantitation of the band intensity is done as outlined in Vandenburgh and Kaufman (29) but using an Helena Cliniscan densitometer in transmittance mode at 590 nm. Myosin heavy chain standards (rabbit muscle, PAGE grade) were obtained from Sigma Chemical Co. At least five different concentrations of myosin heavy chain standard are run on each gel along with the samples of unknown concentrations. Linear regression analysis of the transmittance of the standards on the gel indicated linearity (correlation coefficient = 0.995) in the 1.5 to 10 μg protein range.

Light and Electron Microscopy

Cultures are fixed at varying times with either acetic acid:ethanol (1:3) or buffered 10% formalin, dehydrated with ethanol and stained with Harris hematoxyin/eosin (26) for light microscopic observations. Areas from the formalin-fixed cultures which are to be observed by electron microscopy are embedded in Spurr Embedding Medium (Polysciences, King of Prussia, PA). Thin sections are cut on a Porter Blum MT2B microtome, stained with lead citrate and uranyl acetate, and observed with a Zeiss 9S2 electron microscope.

Results:

When cultured skeletal myotubes differentiated on a collagen-coated monolayer become increasingly more contractile with age, they detach from the substratum either by pulling off from the periphery of the dish as a "sheet" of cells or by rupturing as individual cells. With our standard high density cultures, the myotubes usually begin to detach on day 5 or 6 postplating and this release of mechanical tension progressively continues until the majority of the cells still remaining in the dish are under little tension by 2 weeks. The density of fibroblasts surrounding the myotubes seems to determine whether the cells detach singly or as a sheet, probably because of their collagen-producing ability. The greater the number of fibroblasts in the culture, the better the myotubes are interconnected and the more likely they are to detach together as a sheet (Vandenburgh, unpublished observations). If the cultures are maintained in minimal medium, fibroblast proliferation does not occur, and the myotubes rupture individually. Based on these observations, it seemed likely that by embedding the formed myotubes in a collagen gel matrix and using a peripheral support structure, the contractile cells might be maintained under tension for an extended period of time, even possibly in basal medium.

When the differentiated myotubes are embedded in a collagen gel (collagel) soon after they have formed, the cells appear healthy under the inverted phase microscope and can be kept attached and under tension for at least 2-3 weeks in culture while undergoing strenuous mechanical activity. With continued spontaneous contractions of the embedded myotubes in collagel, the gel dehydrates over a period of 3-4 days, collapsing onto the monolayer of cells. This appears to form a strong supportive collagen mesh over the

cells. This gel dehydration and collapse is similar to what occurs with other mechanically active cells (2). After several weeks in culture, the enmeshed myotubes are no longer attached to the bottom of the wells, but form a tense "floating" sheet of myofibers attached solely to the circular nylon ring or surrounding stainless steel mesh.

Light microscopic observations of the cultures after 2-3 weeks in collagel show them to contain a high density of well-differentiated myotubes of varying diameters (Figure 1). The proliferating fibroblasts form a confluent layer of cells in between the myotubes if the cultures are maintained with complete 85/10/5 medium; in basal medium, fewer fibroblasts are found (Figure 1). Some fibroblasts appear to migrate into the collagel and are seen in a plane above the original monolayer.

The myotubes synthesize and organize myofibrillar proteins into myofilaments as long as they remain attached to the substratum and are under mechanical tension (29), but once the mechanical tension has been released from the myotubes, they develop vacuoles and begin to degenerate when observed by inverted phase microscopy and light microscopy (Figures 1 and 2). Embedding the cells in collagel prevents this cell detachment and degeneration from occurring. As the collagel-embedded myotubes fill with myofibrils, many of their nuclei become localized under the sarcolemma (Figure 2), a characteristic of a more mature muscle cell. When the myotubes are differentiated in complete medium, collagen-embedded, and then maintained in basal medium without supplements, myotube structure, and myofibrillar organization can be maintained for at least 10 days in these cells as long as they remain under tension i.e. attached (Figure 2).

When the collagel-embedded myotubes are examined by electron microscopy, the muscle cells show a number of characteristics of well-differentiated neonatal myofibers (17,24). These characteristics include a high concentration of organized myofibrillar material in the central, as well as peripheral, portion of the myofibers (Figure 3B); the resultant displacement of the central nuclei in immature myotubes towards the peripheral position found in older in vivo myofibers (Figure 3C); and a more highly differentiated external lamina layer (Figure 3D). Few, if any vacuoles indicative of degenerating cells are found in these myofibers. In contrast, control myotubes grown by standard tissue culture techniques are highly vacuolated and contain disorganized myofibrillar material (Figure 3A), because they are no longer under tension in the cultures.

Total cellular protein and DNA content of control and collagel-embedded cultures were measured at varying times after plating and the results of a typical experiment are shown in Figure 4. Total cell protein increases in both cultures from day 1 to day 5 postplating but this level begins to decline in the controls after day 5 (Figure 4A). In contrast, the collagel-embedded cells continue to accumulate protein for 2-3 additional days and are able to maintain this elevated protein level for at least several weeks. The accumulated level of protein in the collagel cultures is 3-4 times the level found in control cultures. Cell density in the cultures as measured by total cell DNA content is greatest at Day 3 in controls, and steadily decreases for 4-6 days,

reaching a plateau for the subsequent 1-2 weeks in culture (Figure 5B). The collagel-embedded cultures show a 20-30% increase in DNA content during the period of DNA decreases in control cultures, due mainly to the continued proliferation of fibroblasts. When the cultures are observed by inverted phase microscopy, this loss in protein and DNA content appears to result partially from the complete detachment of some myotubes and fibroblasts, but mainly from the degeneration of the many partially detached myotubes and fibroblasts remaining in culture. When the protein to DNA ratios are calculated for control and collagel-embedded cultures, the resultant data (Table I) indicate that the cells remaining in the collagel-embedded cultures have a significantly higher ratio than control cells. A higher ratio indicates that the cells in the collagel-embedded cultures are larger cells then in the control cultures, supporting the microscopic observations that the cells in the control cultures are degenerating. Subsequent experiments carried out for longer periods of time (3-4 weeks) indicate that similar trends are maintained for longer periods of time in culture (data not shown).

Composition of the medium has an important influence on the level of spontaneous contractility of the myotubes, their subsequent growth and attachment to the substratum. There is a noticeable variation in the level of long-term myotube spontaneous contractions between different batches of horse serum and chick embryo extract when the cells are embedded in collagel (Vandenburgh, unpublished observations), In our normal medium containing 10% horse serum and 5% chicken embryo extract, moderate contractility of the myotubes occurs during the 2-3 week period of the experiments described in this paper. In the collagel-embedded cultures, the cells remain under tension and are well attached to the nylon ring or stainless steel mesh. Decreasing the percent of horse serum and chick embryo extract in the medium appears to decrease the level of contractile activity when the cells are observed by inverted phase microscopy. Their growth is also reduced in this medium (Figure 5).

When the cells are in basal medium containing horse serum as the only additive, growth is stimulated over basal medium without supplements along with the level of spontaneous contractions; but not as much as when the cells are in medium containing both serum and chicken embryo extract (Figure 5). Growth of the myotubes in medium containing only chicken embryo extract as a supplement leads to violent contractions of the cells and their partial detachment as a sheet even in the collagel-embedded cultures after several days. When the ratio of total protein to DNA content of the cells is calculated, it is evident that increasing concentrations of horse and embryo extract together leads to the best growth of cells, as indicated by the higher ratio values (Figure 6).

Myosin heavy chain content of the cultures under varying growth conditions was measured in control and collagel-embedded cultures. Results of a typical experiment are given in Table II. This experiment has been repeated three times with similar results. The myotubes embedded in collagel accumulate myosin in 2-3 times greater quantity than control non-collagel embedded cultures. This agrees with the light and electron microscopic observations (Figure 3) indicating that the cells embedded in collagel contains greater amounts of organized myofibrillar material than non-embedded controls.

Discussion:

Collagen and other extracellular matrix components play an important role in the differentiation and development of many cell types (14,18). When tissue-cultured cells are embedded in three-dimensional collagen matrices, they are able to differentiate into more in vivo-like tissues (2,6,13,31). For skeletal muscle cells, collagen has been known for many years to have an important influence on myotube formation from single precursor myoblasts (18, 19). We have utilized the collagen-embedding (collagel) technique, coupled with the addition of a supportive surrounding ring structure (artificial "tendon"), to enhance the long-term survival and growth of highly contractile myotubes into "neonatal-like" myofibers. This culturing technique appears to allow better attachment and growth of the myotubes as indicated by inverted phase, light and electron microscopic observations, as well as by the greater total cell protein and DNA accumulation and higher protein/DNA ratios when compared to standard non-collagel culturing techniques. The increase in accumulation of cell protein and DNA in the collagel cultures appears to be the result of two factors. First, under normal culturing techniques, some of the spontaneously contractile myotubes completely detach from the substratum and are lost into the medium. Since increased myotube contractility occurs as the cells mature, this process results in the loss of the most mature myotubes from the cultures. Second, many of the myotubes only partially detach from the substratum, either as a sheet of cells or singly. This results in the release of tension on the myotubes, which is known to be an important growth regulator in the cultured cells (29). The collagel technique prevents both of these processes from occurring.

No attempt was made in our experiments to remove the accompanying fibroblasts which continue to proliferate in the cultures and invade into the collagel matrix. We allowed fibroblasts to remain in the cultures for several reasons. First, they may provide a better environment for the myotubes to develop by synthesizing growth factors (30) or extracellular matrix components not originally in the collagel or produced by the muscle cells themselves. Secondly, the addition of the DNA polymerase inhibitor cytosine arabinoside to remove proliferating cells from the postmitotic myotube cultures always leads to less healthy-looking myotubes, based on vacuoles seen in the cells by phase contrast microscopy (Vandenburgh, unpublished observations). Whether this is due to a direct effect of the drug on the muscle cells, or is due to the removal of proliferating cells which are necessary for healthier myotubes, is not known.

In the light and electron microscope, the collagel-embedded myotubes take on several of the morphological characteristics of immature neonatal myofibers, such as a well-developed external lamina, peripherally located myonuclei, and well organized myofilamentous material. It has been difficult in other culture systems to obtain neonatal-like myofiber differentiation from primary myoblasts. The more complete external lamina development in the collagel cultures is not surprising in light of the observations that many components of the external lamina are synthesized and secreted into the medium of cultured myotubes rather than being incorporated into the cell's immediate surrounding environment (10). Fibronectin, one component of the external lamina, is synthesized and secreted in muscle cultures and the fraction of the secreted fibronectin which appears to be incorporated into the external lamina of the cells occurs mainly at the collagen substratum-cell membrane interface (12). It is therefore, not unreasonable to expect that by completely surround-

ing the myotubes by a collagen matrix, stabilization and incorporation of fibronectin and possibly other external lamina components will occur around the entire periphery of the cells.

The immature myotube's nuclei both in vivo and in vitro normally occupy a position in the central region of the cells' cytoplasm (24). With differentiation there is an increasing incorporation of muscle-specific proteins into myofilaments and their filling of the myotube's cytoplasm, usually fom the periphery inward. This is accompanied by many of the myotube's nuclei being relocated into a subsarcolemmal position. Under normal tissue culture conditions, most of the myotube's nuclei remain in the cell's interior. In contrast, when the myotubes are grown in collagel, many of the nuclei are subsequently relocated to a subsarcolemmal position after 1-2 weeks in culture. This relocation is probably initiated by the increased appearance of organized myofibrillary material in the collagel-embedded myotubes, as seen by electron microscopy. The appearance of satellite-like cells along the periphery of the myofibers in in vivo is also a characteristic of a more mature myofiber. Since we did not perform electron microscopy on these cells in the present study, we do not know if cells which fit into the classical definition of a satellite cell (i.e. located within the external lamina of the myofiber) are occurring in the collagel cultures. Phase and light microscopy show many cells closely opposed to the myofiber (Figures 1 and 2) but whether they have a distinct membrane separating them from the myofiber's cytoplasm will require further study.

Quantitation of one of the myofibrillar proteins, the myosin heavy chain, by polyacrylamide gel electrophoresis, supports the observations from electron microscopy that greater levels of myofilaments appear in the collagel-embedded cultures. The collagel-embedded cells are able to accumulate 2-3 times more

myosin heavy chain protein than normal cultures grown on a collagen-coated substratum. This improved accumulation of myosin is partly due to fewer detached cells, along with better differentiation of the remaining attached myofibers kept under tension in the collagel-embedded cultures. The better myosin accumulation in the collagel cultures is not surprising, considering the known importance of tension in regulating the levels of myosin both in vitro (29) and in vivo (1,16).

Experiments performed in media of several different compositions indicate that the collagel-embedded cell culture technique will be useful for the analysis of the role of horse serum and chick embryo extract factors in myotube differentiation into more mature myofibers. This culture system, by maintaining the highly contractile cells in a growth state and under tension, even in less-enriched media, should also be useful in the analysis of the various defined growth factors on the long-term tissue growth. Addition of medium supplements increases the cell's growth, as evidenced by the increasing total cell protein and DNA levels. More complete analysis is needed to determine the various roles played by serum and embryo extract factor in the more complete differentiation of the muscle cells in the collagel system. In analyzing the influence of various medium growth factors on the myofiber's growth, some consideration will have to be given to the differences in spontaneous contractile activity which are observed in media of various compositions, since activity is a very important regulator of protein turnover in skeletal muscle (33). Differentiation between a growth factor's effects on myofiber growth due to direct hormone-receptor interactions versus indirect mechanical activity effects should be possible, since drugs such as tetrodotoxin can be used to inhibit muscle mechanical activity.

In summary, the new culture method described in this paper appears to be

an improved culturing technique for the in vitro analysis of the differentiation of immature myotubes into neonatal-like myofibers under highly regulatable tissue-culture conditions. This culturing technique, when coupled to other in vivo-like influences on muscle growth which are lost in normal culturing techniques such as innervation, and mechanical activity, may prove to be a useful tool for the analysis of the factors involved in skeletal muscle growth and differentiation to the adult stage.

Acknowledements:

We would like to thank Dr. Sumner Zacks for critical reading of the manuscript, Xia Xiong, Pat Karlisch, and John Monroe for technical assistance; Kathleen King and Del Poulin for typing the manuscript. This work was supported by NIH Grant AM 36266 and NASA Research Grant NAG2-414.

TABLE I

Total Cell Protein/DNA Ratios of the Cultured Muscle Cells

Days in Culture	Control Cultures Mean ± S.D. (N = 2-4)	Collagel Cultures Mean ± S.D. (N = 2-4)
3	18.8 ± 0.1	17.5 ± 0.3
5	29.3 ± 4.3	37.4 ± 4.2
8	35.3 ± 6.4	46.8 ± 3.4
15	28.9 + 7.2	35.3 + 1.0
Mean of Days 5-15	31.2 ± 3.6	39.8 ± 6.1*

*Days 5-15 control vs. collagel culture ratios are significantly different at the $p < .03$ level based on a paired t-test.

Embryonic avian myoblasts were plated onto collagen-coated tissue culture dishes containing nylon ring supports as described in Methods. The cells differentiated into contractile myotubes by Day 3 postplating. At that time, half the cells were embedded in a collagel matrix; all the cultures were fed fresh medium (85/10/5) every third day thereafter. At the times indicated, the cultures were processed for total cell protein and DNA content as outlined in Methods.

TABLE II

Myosin Heavy Chain Accumulation in Skeletal Myotube Cultures

Days in Culture	N	Controls (μg/dish $\pm$ SD)	Collagel (μg/dish + SD)	p value
8	4	23 $\pm$ 8	43 $\pm$ 13	$p < .05$
15	3	22 $\pm$ 7	65 $\pm$ 23	$p < .05$

N = number per group

P value using unpaired t-test

This experiment was performed as outlined in Table I Legend. Myosin heavy chain accumulation was determined by densitometric scanning of the myosin heavy chain band isolated by the polyacrylamide gel electrophoresis method of Paterson and Strohman (25). Five different concentrations of myosin heavy chain standards (Sigma) were run with each group of cultured muscle protein samples.

Figure Legends:

Figure 1. Skeletal myotubes after 17 days in culture. The cells were grown for the first 72 hours in 85/10/5 medium, and then maintained in either 85/10/5 medium (A, B) or Basal Medium (Eagle's) without supplements (C, D). A and C are control cultures, B and D collagen-embedded cultures. Arrowheads indicate partially detached myotubes under little tension.H & E stain, x 125.

Figure 2. Light micrographs of skeletal myotubes after 17 days in culture. The cells were grown for 72 hours in 85/10/5 medium and then main- tained in Basal Medium (Eagle's) without supplements. A. Control cultures, B. collagel cultures. Striations are clearly visible in larger myotubes with subsarcolemmal nuclei in the collagel-embedded cells. Arrowheads indicate partially detached myotubes under little tension. H & E stain x 1000.

Figure 3. Electron micrographs of 18 day cultured myotubes. A. Control cells are vacuolated, and contain loosely organized myofibrillar material (arrowheads). B, C, D. Collagel-embedded cells which have characteristics of neonatal myofibers including well organized myofibrillar material (B), a peripherally migrating nucleus (C), and well developed external lamina (arrowhead) (D). Magnifications are A. 3,000; B. 9,000; C. 9,000; D. 4,000.

Figure 4. Total cell protein (A) and DNA (B) accumulation in control and collagel cultures at varying times after plating. The collagen gel procedure was performed on Day 3, with approximately 40 μg collagen protein added to each of the collagel cultures. Values are mean $\pm$ SD of 2-4 cultures.

Figure 5. Effects of changing the composition of the medium on total cell protein and DNA accumulation in collagel cultures. All cells were fed 85/10/5 media up to Day 5, at which time fresh media of varying compositions were added every third day as indicated on the x axis for the subsequent days (18)

of the experiment. Values are the means ± SD of 2-4 cultures. The absence of S.D. bars from some of the data points indicates that the bars were smaller than the data points. Total cell protein accumulation (○). Total cell DNA content (●). Age-matched control cultures not embedded in collagel had total cell protein content of 99 ± 35 and DNA content of 4.4 ± 2.3 g/dish.

Figure 6. Ratio of total cell protein/DNA content of the cells from the experiment outlined in Figure 5. Each bar is the mean ± SD of 2-4 values.

References:

1. Ashmore, C.R., Summers, P.J. Stretch-induced growth in chicken wing muscles: Myofibrillar proliferation. Am. J. Physiol. 51:C93-C97, 1981.

2. Bell, E., Ivarsson, B., Merrill, C. Production of tissue-like structure by contraction of collagen lattices by human fibroblasts of different proliferative potential in vitro. Proc. Natl. Acad. Sci USA 76:1274-1278, 1979.

3. Buckingham, M. Muscle cells in tissue culture. Int. Rev. Biochem. 15: 315-322, 1977.

4. Buckingham, M.E. Actin and myosin multigene families: Their expression during the formation of skeletal muscle. Essays in Biochem. 20:77-109, 1985.

5. Caplan, A.I., Fitzman, M.Y., Eppenberger, H.M. Molecular and cell isoforms during development. Science 221:921-927, 1983.

6. Chambard, M., Gabron, J., Mauchamp, J. Influence of collagen gel on the orientation of epithelial cell polarity: Follicle formation from isolated thyroid cells and from preformed monolayers. J. Cell Biol. 91:157-166, 1981.

7. Coleman, J.R., Coleman, A.W. Muscle differentiation and macromolecular synthesis. J. Cell. Physiol. 72:19-34, 1968.

8. de la Haba G., Kamali, H.M., Tiede, D.M. Myogenesis of avian striated muscle in vitro: Role of collagen in myofiber formation. Proc. Natl. Acad. Sci. 72:2729-2732, 1975.

9. Ecob, M., Butler-Browne, G.S., Whalen, R.G. The adult fast isozyme of myosin is present in a nerve-muscle tissue culture system. Differentiation 25:84-87, 1983.

10. Fambrough, D.M, Bayne, E.K., Gardner, J.M., Anderson, M.J., Wakshull E., Rotundo, R.L. Monoclonal antibodies to skeletal muscle cell surface in Neuroimmunology, Brockes, J. ed., Plenum Press, pp. 49-89, 1982.

11. Fischman, D.A. The Structure and Function of Muscle. Bourne, G.H. ed. Vol. 1, New York: Academic Press, pp 75-148, 1973.

12. Gardner, J.M., Fambrough D. Fibronectin expression during myogenesis. J. Cell Biol. 96:474-485, 1983.

13. Hall, H.G., Farson, D.A., Bissell, M.J. Human formation by epithelial cell lines in response to collagen overlay: A morphogenetic model in culture. Proc. Natl. Acad. Sci. 79:4672-4676, 1982.

14. Hay, E.D., ed. Cell Biology of the Extracellular Matrix, Plenum Press, New York, 1982.

15. Hauschka S.D. and Konigsberg, I.R. The influence of collagen on the development of muscle clones. Proc. Natl. Acad. Sci (U.S.A.) 55:119-126, 1966.

16. Jablecki, C., Kaufman, S. Myosin adenosine triphosphatase activity during work-induced growth of slow and fast skeletal muscle in the normal rat. J. Biol. Chem. 248:1056-1062, 1973.

17. Kelly, A.M. and Zacks, S.I. The histogenesis of rat intercostal muscle. J. Cell Biol. 42:135-153, 1969.

18. Kleinman, H.K., Klebe, R.J., Martin, G.R. Role of collagenous matrices in the adhesion and growth of cells. J. Cell. Biol. 88:473-485, 1981.

19. Konigsberg, I.R., McElvain, N., Tootle, M., Herman, H. The dissociability of DNA synthesis from the development of multinuclearity of muscle cell in culture. J. Biophys. Biochem. Cytol. 8:333-343, 1960.

20. Konigsberg, I.R. Diffusion-mediated control of myoblast fusion. Develop. Biol. 26:133-152, 1971.

21. Labarca, C., Paigen, K. A simple rapid and sensitive DNA assay procedure. Anal. Biochem. 102:344-352, 1980.

22. Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275, 1954.

23. Moss, P.S. Spector, D.H., Glass, C.A. Strohman, R.C. Streptomycin retards the phenotypic maturation of chick myogenic cells. In Vitro 20:473-478, 1984.

24. Murray, M.R. Skeletal muscle in culture in Structure and Function of Muscle. Vol. I, Part I, Bourne, G.H. ed., New York: Academic Press, pp. 237-299, 1972.

25. Paterson, B. and Strohman, R.C. Myosin synthesis in cultures of differentiating chicken embryo skeletal muscle. Develop. Biol. 29:113-138, 1972.

26. Sheehan, D.C. and Hrapchak, B.B. Theory and Practice of Histochemistry. 2nd ed., St. Louis: C.V. Mosby Co., p. 143, 1980.

27. Stockdale, F.E., Holtzer, H. DNA synthesis and myogenesis. Exp. Cell Res. 24:508-520, 1961.

28. Vandenburgh, H.H. Separation of plasma membrane markers by glycerol-induced blistering of muscle cells. Biochem. Biopshy. Acta 466:302-314, 1977.

29. Vandenburgh, H.H., Kaufman, S. In vitro model for stretch-induced hypertrophy of skeletal muscle. Science 203:265-268, 1979.

30. Vandenburgh, H.H. Cell shape and growth regulation in skeletal muscle: exogenous versus endogenous factors. J. Cell. Physiol. 116:363-371, 1983.

31. Vandenburgh, H.H. Relationship of muscle growth in vitro to sodium pump activity and transmembrane potential. J. Cell. Physiol. 119:283-295, 1984.

32. Walker, C., Strohman, R. Myosin turnover in cultured muscle fibers relaxed by tetrodotoxin. Exp. Cell. Res. 116:341-348, 1978.

33. Waterlow, J.C., Garlick, P.J., Millward, D.J. (Editors) Protein Turnover in Mammalian Tissues and in the Whole Body. Amsterdam, Netherlands: North-Holland, pp. 529-594, 1978.

34. Yang, J., Richards, J., Bowman, P., Guzman, R., Enami, J., McCormick, K., Hamamoto, S., Pitelka, D., Nandi, S. Sustained growth and three-dimensional organization of primary mammary tumor epithelial cells embedded in collagen gels. Proc. Natl. Acad. Sci. (U.S.A.) 76:3401-3405, 1979.

Figure 1

Figure 2.

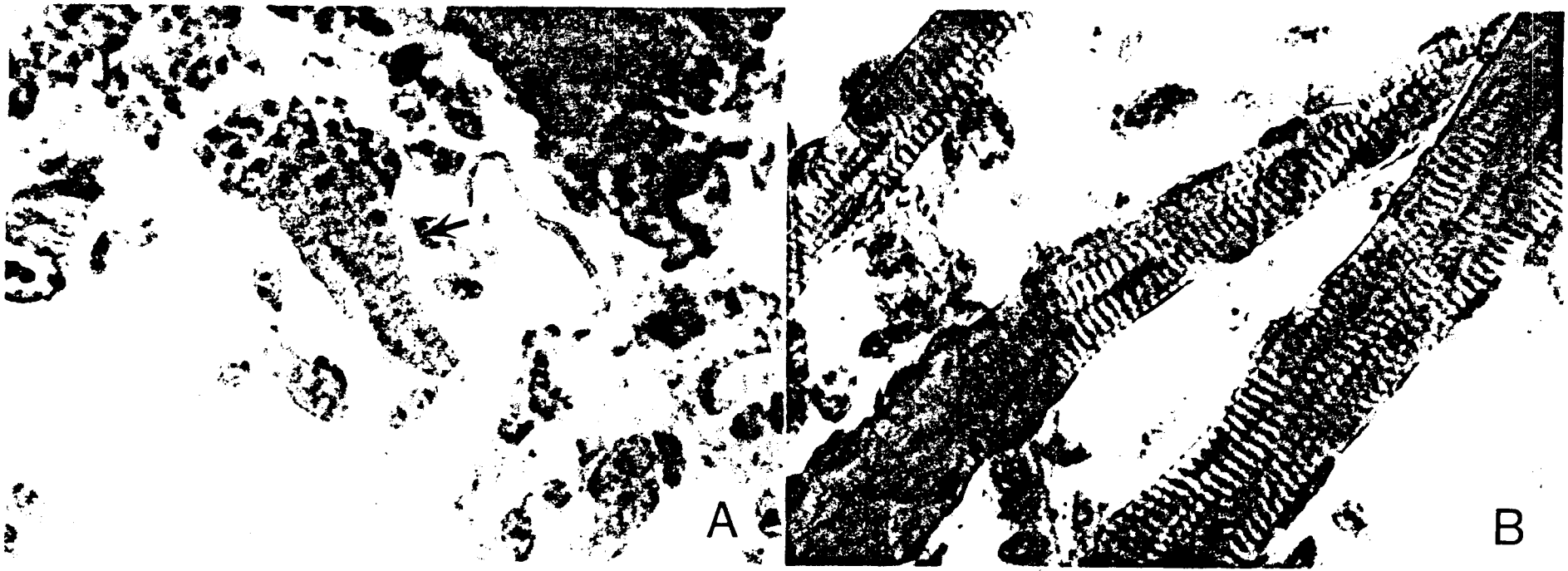


Figure 3

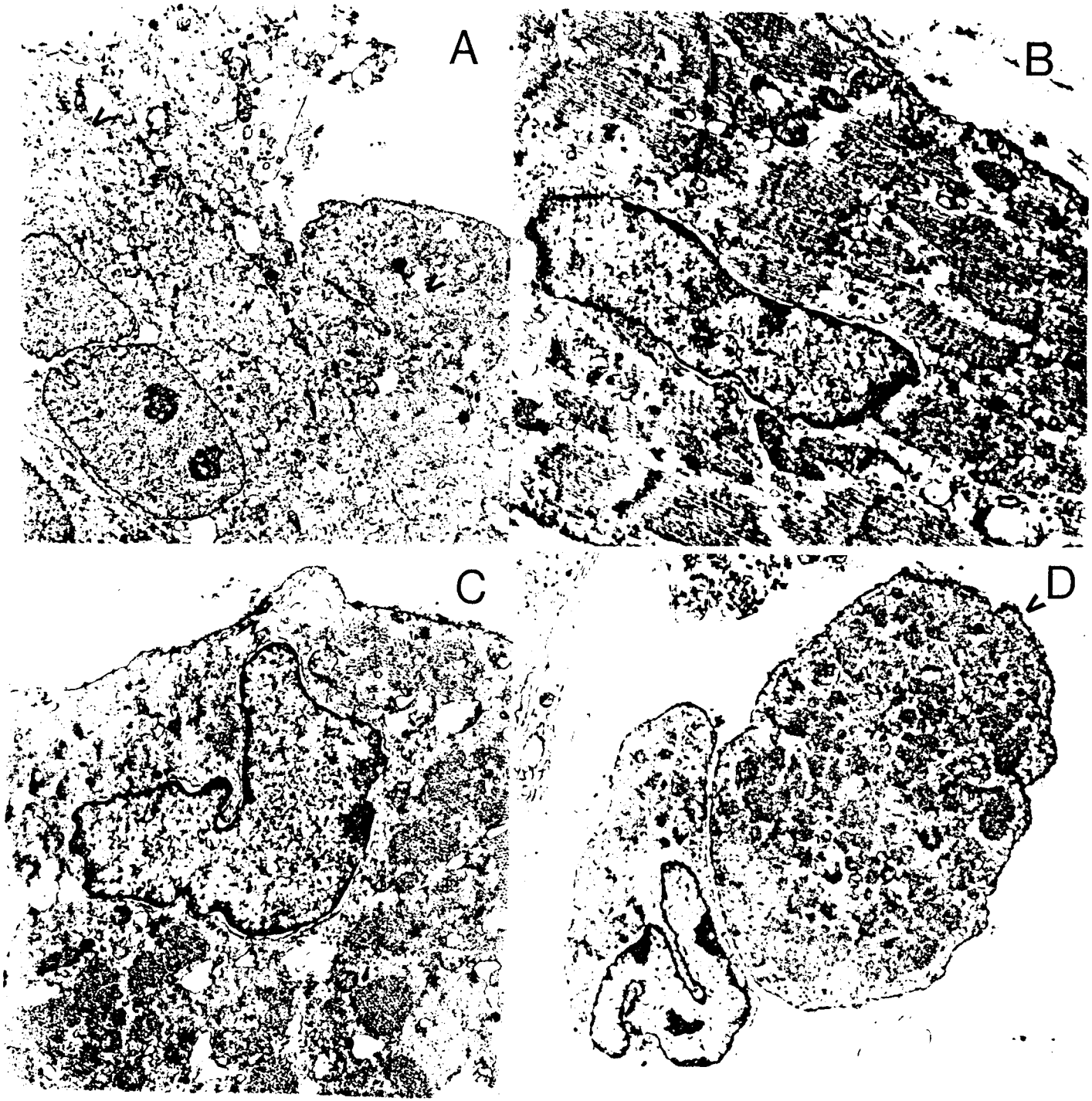


Figure 4B

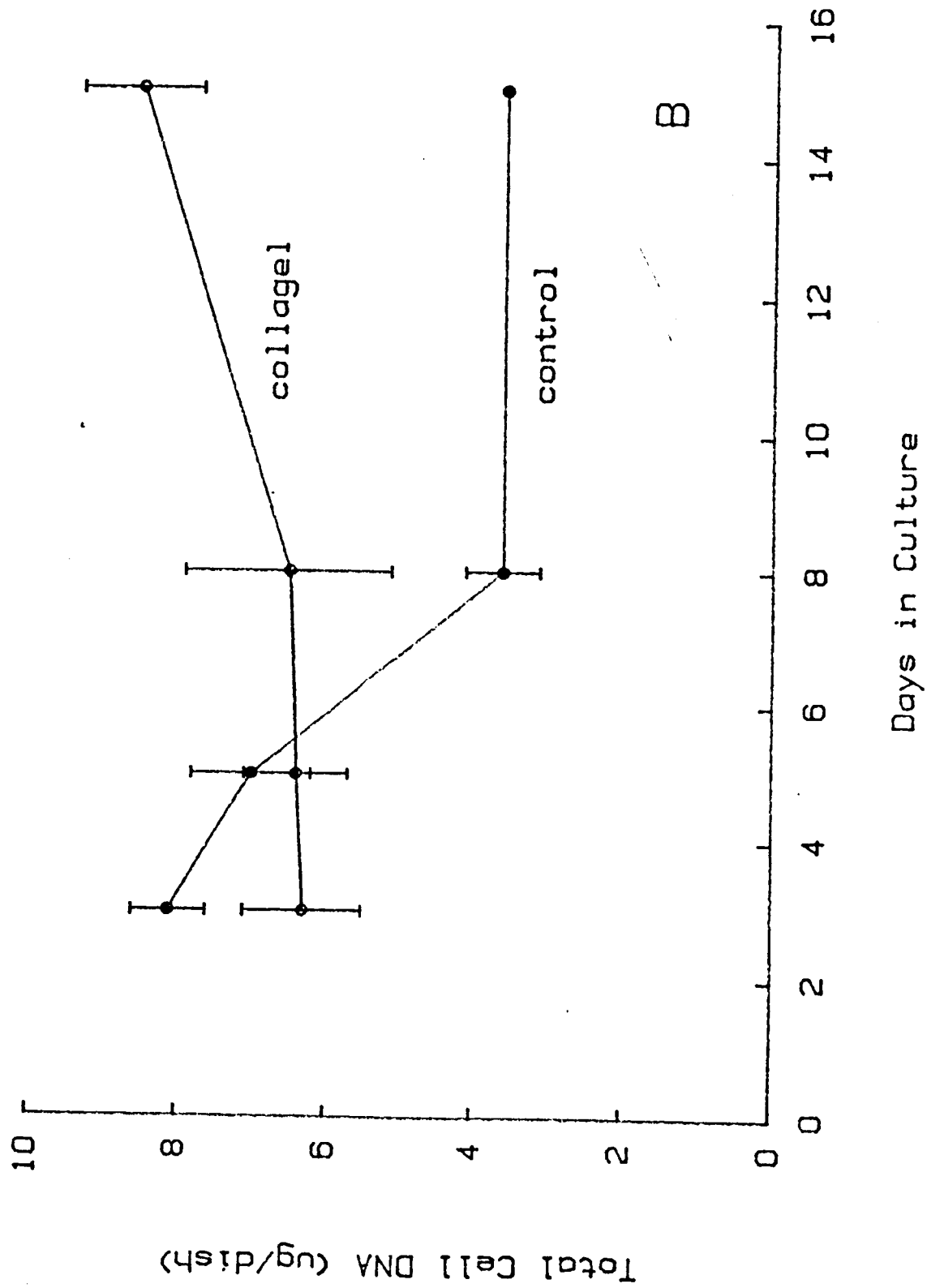

Total Cell DNA (ug/dish)
10
8
6
4
2
0
0
2
4
6
8
10
12
14
16
Days in Culture
collagel
control
B

Figure 5

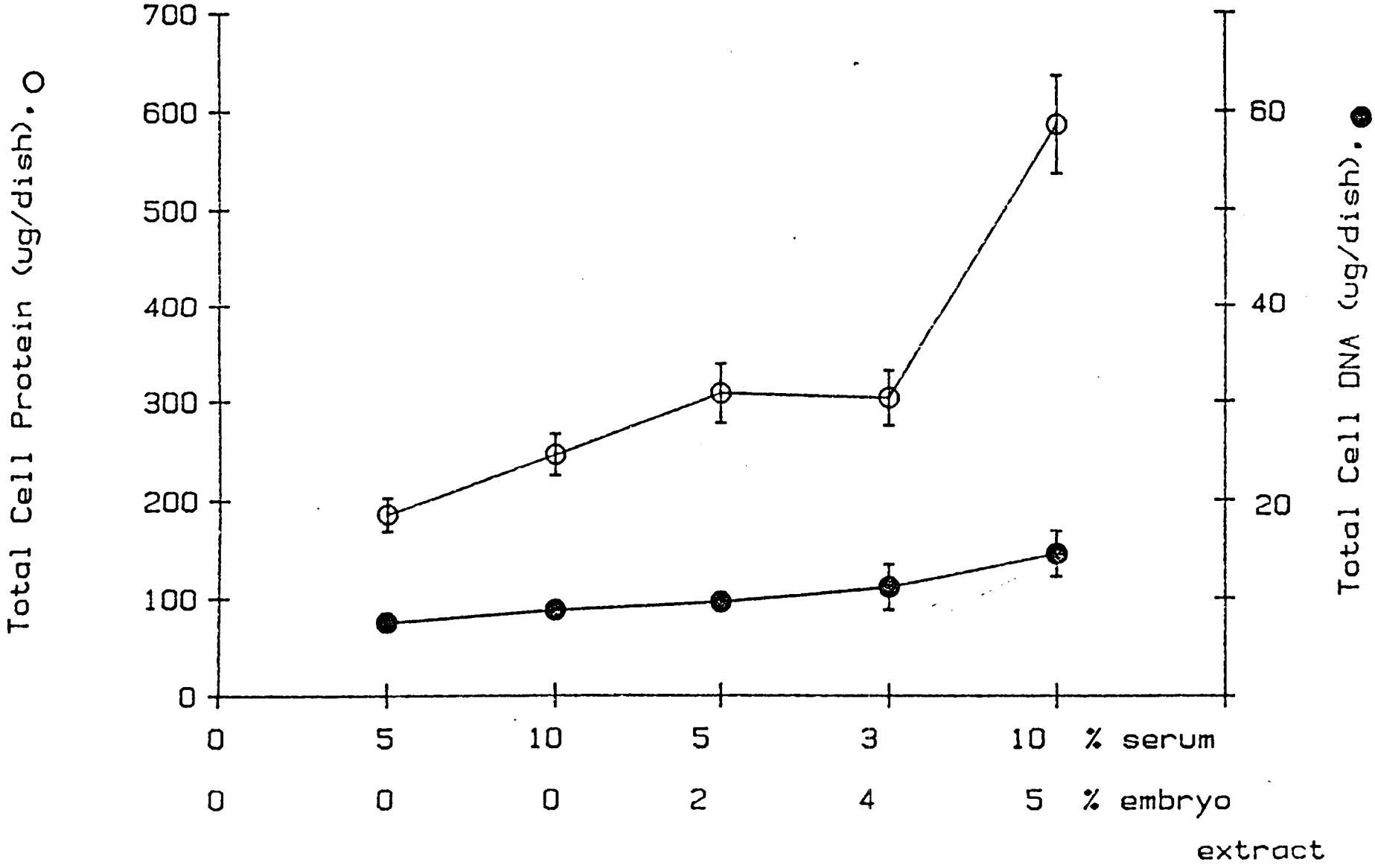
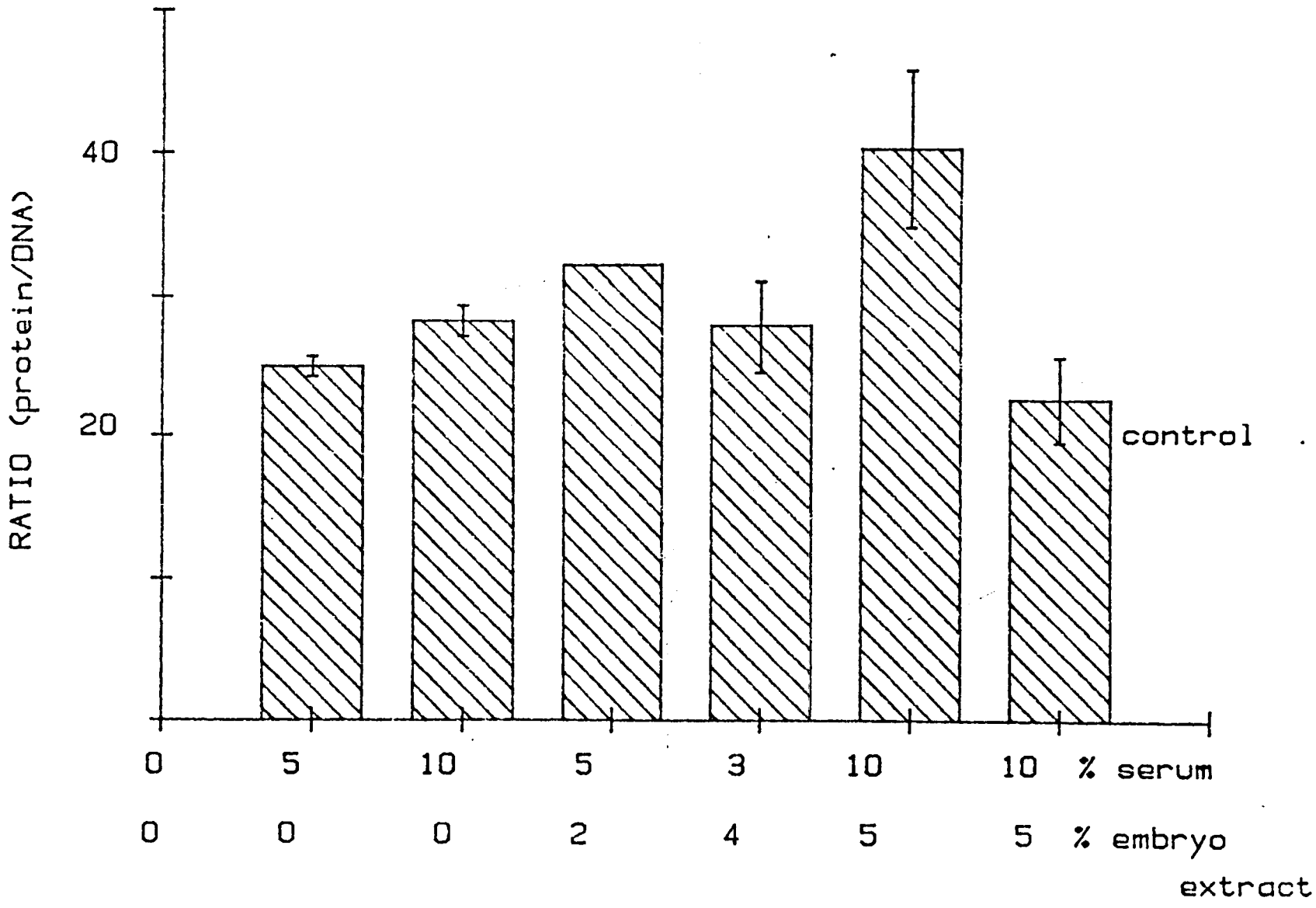

Total Cell Protein (ug/dish). O
700
600
500
400
300
200
100
0
60
40
20
Total Cell DNA (ug/dish). ●
0 5 10 5 3 10 % serum
0 0 0 2 4 5 % embryo extract

Figure 6.


RATIO (protein/DNA)
40
20
control
0
5
10
5
3
10
10
% serum
0
0
0
2
4
5
5
% embryo extract

IN VITRO
cellular & developmental biology

VOLUME 23 NUMBER 3 ISSN 0883-8364 MARCH 1987

PART II

AIAA

PROGRAM ISSUE

THIRTY-EIGHTH ANNUAL MEETING of the TISSUE CULTURE ASSOCIATION

MAY 27-30, 1987

THE HYATT REGENCY CRYSTAL CITY HOTEL WASHINGTON, DC (Arlington, VA)

JOURNAL OF THE TISSUE CULTURE ASSOCIATION

In vitro cellular & developmental biology. VOLUME 23, NUMBER 3, PART II, PAGES 1A-80A, MARCH 1987

49 Normal cell extracellular matrix promotes tumor cell adhesion; comparison with human native basement membrane. R.G. RUSSO*, L. Marmonti, S. Garbisa, (RGR) Boehringer Biochemia Robin, Lab. Pharmacology, 20126 MILAN, (SG) Inst. Histol. Gen. Embryol., University of Studies, 35100 PADUA, ITALY

Cell attachment is presumably the first step in the complex mechanism of metastasis. Tumor cells "chose", in fact, after their dissemination in the body, the favourite site of adhesion (e.g. an extracellular matrix, ECM) to form the metastatic site for subsequent growth and invasion. A panel of human tumor cells (including ovarian and colon carcinomas, a fibrosarcoma, a glioblastoma, a melanoma and a leukemia) were tested on different substrates for their attachment. Initial seeding concentration was 2×10^5 cells/2 ml in each well (3.5 cm diameter). Recovered cells varied between 3×10^4 and 3×10^5 within 24 hrs of growth. Comparison with plastic substrates showed that the cells attached and grew better on fresh ECM prepared from murine embryo fibroblasts (3T3), or human amnion epithelium (FL-A), or bovine endothelium (BAE) grown to confluence. SDS-PA gel electrophoresis showed differences among ECMs produced by 3 normal cell lines and results have been correlated with type I and type II collagens, fibronectin and laminin contents. Human amnion basement membrane (prepared as in R.G. Russo et al., Clin. Expl. Metast., 1:115, 1983) has been employed as a reference native epithelial substrate to verify adhesive characteristics of various cell lines. Its biochemical structure has been identified by slab gel electrophoresis.

50 Chick Iris Pigmented Epithelial Cells in vitro. L. SARTORI*, Mark C. Skiba and Christopher Murray, Western New England College, Springfield, MA 01119.

Chick iris pigmented epithelial cells were grown in a medium consisting of MEM and 10% fetal bovine serum. H^3-thymidine incorporation by the cells showed that the cells were not quiescent but undergoing typical cell cycles. The cells grew out from explants in very densely populated sheets. The shapes of the cells were hexagonal with each having a centrally located nucleus. A single protein was isolated from the cell surfaces using 5% SDS-polyacrylamide gel electrophoresis. The protein has an approximate weight of 210,000 daltons.

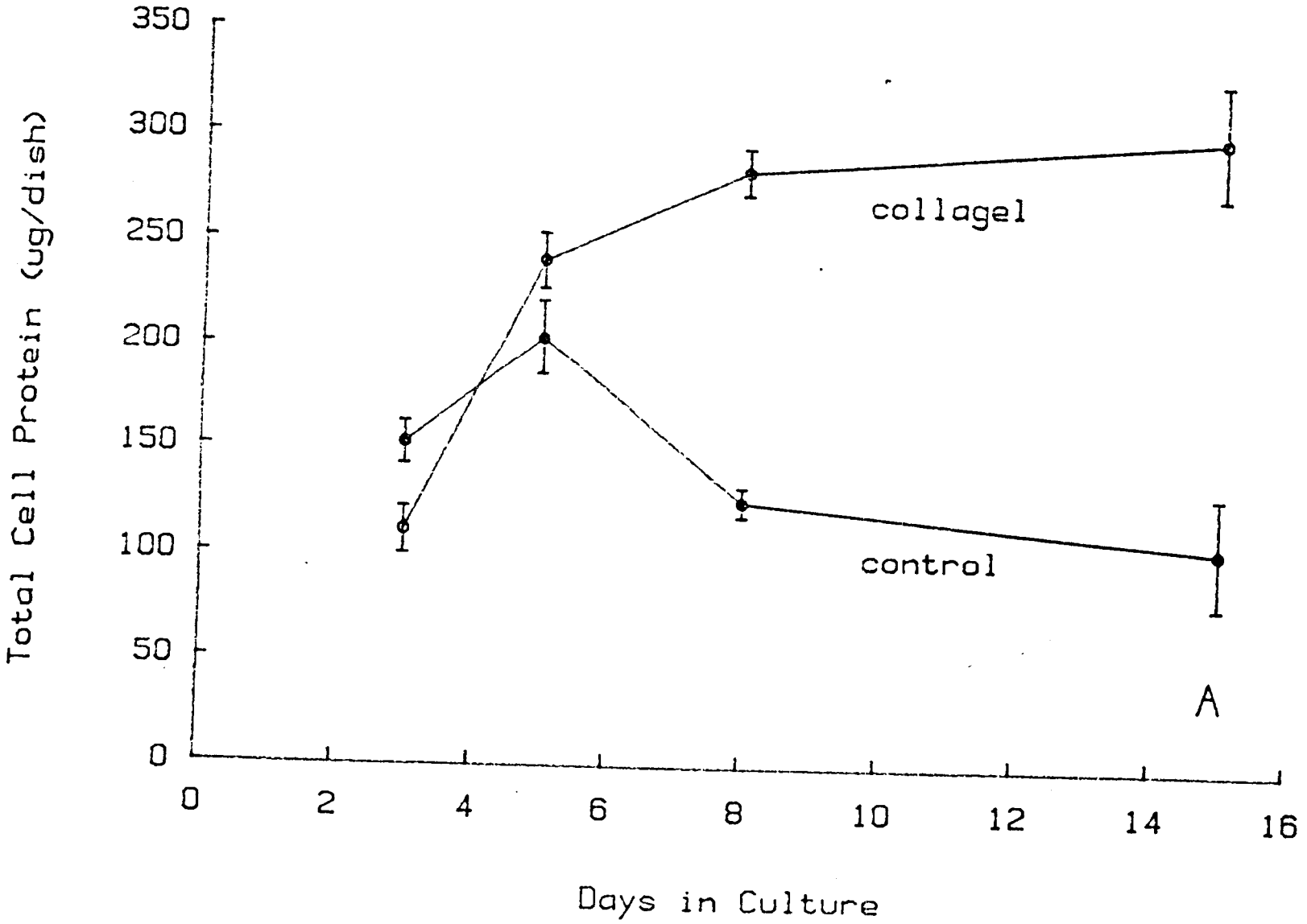
51 Maintenance of Highly Contractile Tissues Cultured Avian Skeletal Myotubes in Collagen Gel. H.H. VANDENBURGH*, P. Karlisch and L. Farr, Department of Pathology, Brown University and Department of Laboratory Medicine, The Miriam Hospital, 164 Summit Avenue, Providence, RI 02906.

Skeletal muscle activity is important for the maintenance of muscle protein content, with inactivity leading to cell atrophy. In tissue culture, highly contractile skeletal myotubes are difficult to maintain in high density on collagen-coated dishes for extended periods of time because of their propensity to detach from the substratum. This detachment results in the release of tension from the cells and the loss of cell protein. A simple method is described for long-term culturing of high density contractile primary avian myotubes embedded in a collagen matrix (collagel) and attached to a circular nylon ring. After several weeks in culture, a diaphragm-like thin layer of myotubes develops which is no longer attached to the culture dish but only to the circular ring. Total cell protein and myosin heavy chain accumulation is maintained in this collagel system at levels which are 2-3 times those found in time-matched controls. Morphologically, the myotubes have the structural characteristics of neonatal myofibers - subsarcolemmal nuclei, well organized myofibrillar and external lamina material. This new collagel culture system should prove useful in examining the relationship of muscle activity to muscle growth and to gene expression during myotube to myofiber differentiation. (Supported by NIH Grant AM 36266 and NASA Research Grant NAG2-414).

52 A Computerized Model System for Studying the Effects of Mechanical Activity on Cell Growth In Vitro. H.H. VANDENBURGH*, Department of Pathology, Brown University and Department of Laboratory Medicine, The Miriam Hospital, 164 Summit Avenue, Providence, RI 02906

Mechanical activity has important growth regulating effects on many tissues, including bone, skeletal, cardiac, and smooth muscle. The molecular mechanism for this physical to biochemical transduction is unknown, but the cytoskeleton is probably vital. To study this transduction, a new model system has been developed in which cultured cells can be grown on an elastic substratum and mechanically stretched and relaxed repetitively to a linear accuracy of 30 m. The controlling unit in the Mechanical Cell Stimulator (MCS) is an Apple IIe computer interfaced with 24 well Cell Growth Chamber through optical data links. The set-up can be placed in a standard CO_2 incubator and the MCS software is capable of simulating many different types of mechanical activity patterns which cells are subjected to in vivo. The Cell Growth Chamber allows easy access for multiple sampling for time course studies. We have utilized this system to show that 1) primary avian myoblasts fuse into myotubes which are oriented parallel to the direction of a continuous stretch in one direction (0.35 mm/hr for 24 hrs) but perpendicular to the direction of repetitive mechanical activity (2 Hertz for 10 sec, 150 sec rest, for 24 hrs); and 2) the myotube's growth rate is stimulated by both types of mechanical activity. (Supported by NIH Grants AM36266 and RR 0518.)

Figure 4A


Total Cell Protein (ug/dish)
350
300
250
200
150
100
50
0
0
2
4
6
8
10
12
14
16
collagel
control
A
Days in Culture